PB84-223023

Causes of Papillomas on Fish Exposed to Chlorinated Sewage Effluent

Auburn Univ., AL

Prepared for

Environmental Research Lab., Gulf Breeze, FL

Jul 84

U.S. Department of Commerce
National Technical Information Service
NTIS

PB84-223023

EPA-600/3-84-076
July 1984

CAUSES OF PAPILLOMAS ON FISH EXPOSED TO CHLORINATED SEWAGE EFFLUENT

by

John M. Grizzle and Paul Melius
Department of Fisheries and Allied Aquacultures
and Department of Chemistry
Auburn University, Alabama 36849

CR809336010

Project Officer

William Davis
Environmental Research Laboratory
Gulf Breeze, Florida 32561

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
GULF BREEZE, FLORIDA 32561

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/3-84-076	2.
3. RECIPIENT'S ACCESSION NO. PB84 223023	
4. TITLE AND SUBTITLE CAUSES OF PAPILLOMAS ON FISH EXPOSED TO CHLORINATED SEWAGE EFFLUENT	5. REPORT DATE July 1984
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) J.M. Grizzle and Paul Melius	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Fisheries and Allied Aquacultures & Department of Chemistry Auburn University, Alabama 36849	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO. CR80933010
12. SPONSORING AGENCY NAME AND ADDRESS U.S. Environmental Protection Agency Environmental Research Laboratory Office of Research & Development Gulf Breeze, FL 32561	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA/600/04
15. SUPPLEMENTARY NOTES	

16. ABSTRACT

This research was initiated to determine the cause of oral papillomas in black bullheads (Ictalurus melas) from the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant. The water in this pond was chlorinated effluent from the sewage treatment plant.

The presence of a carcinogenic and mutagenic chemical in the effluent of a sewage treatment plant was indicated by papillomas developing on caged black bullheads, glucuronosyltransferase induction in caged channel catfish, and Ames-test mutagenicity of water extract. Unlike previously studied fish papillomas, virus-like particles were not present in the tumor cells. Although mutagenic and carcinogenic chemicals have not been identified in the wastewater, chlorine is implicated as a factor contriubting to the induction of the papillomas because the prevalence of papillomas on wild black bullheads exposed to the effluent decreased from 73% to 23% after the chlorination rate was reduced.

This report was submitted in fulfillment of Grant No. LCR809336010 by Auburn University under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period from 12 October 1981 to 11 October 1983, and work was completed as of 11 October 1983.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS (This Report) unclassified	21. NO. OF PAGES 32
	20. SECURITY CLASS (This page)	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

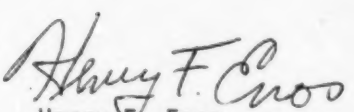
The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

the effects of toxic organic pollutants on individual species and communities of organisms;

the effects of toxic organics on ecosystem processes and components;

the significance of chemical carcinogens in the estuarine and marine environments.

Few studies have reported carcinogenic expressions in populations of fishes, and still fewer researchers have been able to manipulate the frequency of the tumor expression. In this report, a population of catfish exposed to chlorinated sewage effluent are caged and compared with related species, and efforts to separate the tumor inducer were undertaken. Results indicated that the incidence of papillomas declined after the chlorination rate was reduced. These data provide an intriguing link between waste water treatment and tumor induction in fish.



Henry F. Enos
Director
Environmental Research Laboratory
Gulf Breeze, Florida

ABSTRACT

This research was initiated to determine the cause of oral papillomas in black bullheads (*Ictalurus melas*) from the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant. The water in this pond was chlorinated effluent from the sewage treatment plant.

The presence of a carcinogenic and mutagenic chemical in the effluent of a sewage treatment plant was indicated by papillomas developing on caged black bullheads, glucuronosyltransferase induction in caged channel catfish, and Ames-test mutagenicity of water extract. Unlike previously studied fish papillomas, virus-like particles were not present in the tumor cells. Although mutagenic and carcinogenic chemicals have not been identified in the wastewater, chlorine is implicated as a factor contributing to the induction of the papillomas because the prevalence of papillomas on wild black bullheads exposed to the effluent decreased from 73% to 23% after the chlorination rate was reduced.

This report was submitted in fulfillment of Grant No. CR809336010 by Auburn University under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period from 12 October 1981 to 11 October 1983, and work was completed as of 11 October 1983.

CONTENTS

	Page
Abstract	iv
Figures	vi
Tables	vii
Acknowledgments	viii
1. Introduction	1
2. Conclusions and Recommendations	2
3. Study Site	3
4. Materials and Methods	6
5. Results	10
6. Discussion	19
References	21

FIGURES

Number		Page
1	Schematic plan of the Tuskegee, Alabama, sewage treatment plant . . .	4
2	Total residual chlorine (monthly averages) in water leaving the chlorine contact chamber of the Tuskegee sewage treatment plant . .	5
3	Prevalence of papillomas on wild black bullheads from the final oxidation pond of the Tuskegee sewage treatment plant	11
4	Oral papilloma on the mouth fornix of a black bullhead.	12
5	Superficial portion of an oral papilloma. Epithelial cells, goblet cells, and alarm substance cells are arranged around lumens forming cell nests.	13
6	Response of Salmonella typhimurium strain TA100 in the Ames test to organic extracts of water collected at the inlet or outlet of the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant	16
7	UDP-glucuronosyltransferase activity (nmol/min/mg protein)in liver microsomes of caged channel catfish in the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant or in a control pond	18

TABLES

Number		Page
1	Maximum prevalence of oral lesions on caged fish in the final oxidation pond of the Tuskegee sewage treatment plant	15

ACKNOWLEDGMENTS

The cooperation of the South Tuskegee Water Pollution Control Plant personnel, is gratefully acknowledged. Auburn University staff and graduate students that contributed to this project were Barrie Tan, Chalor Limsuwan, Leonard Hochstedler, Paraskevi Karathanasis, Diane Biba, Richard Maxwell, Jan Hosey, and Steve Horowitz.

SECTION 1

INTRODUCTION

A population of black bullheads in the final oxidation pond of the City of Tuskegee, Alabama, sewage treatment plant had a 73% prevalence of oral papillomas (Grizzle et al. 1981). These fish provided an opportunity to study the cause of fish papillomas, especially as related to chlorinated sewage effluent.

Sewage entering this treatment plant did not include discharge from industries or institutions, but run-off water from residential and agricultural areas entered the plant after rains. The process of chlorinating the sewage after it passed through an aeration basin and a settling basin was considered a possible source of carcinogens.

There has been only one previous report of a high prevalence of neoplasms in aquatic animals living in chlorinated sewage effluent (Rose 1977). In that case, a pond at Reese Air Force Base, Texas, contained tiger salamanders (Ambystoma tigrinum) that had neoplastic and non-neoplastic lesions with a peak frequency of 53%. Chemical carcinogens, especially perylene, were suspected of causing the lesions.

Papillomas occur on many species of fish and have been associated with pollution (Lucke and Schlumberger 1941; Russell and Kotin 1957; Cooper and Keller 1969; Sonstegard 1977; Dawe and Harshbarger 1975) and tumorogenic viruses (Edwards et al. 1977; Schwanz-Pfitzner 1976; McAllister et al. 1977). Other fish tumors have a genetic origin (Siciliano et al. 1971; Vielkind et al. 1971).

Laboratory and field experiments were initiated to determine the cause of the papillomas. Some of these experiments were a continuation of a previous EPA cooperative agreement (CR807844010, Grizzle and Melius 1983). Objectives were to: (1) determine the prevalence of papillomas on fish in the final oxidation pond; (2) describe the histology and ultrastructure of the papillomas; (3) determine if the papillomas would persist on black bullheads taken from the final oxidation pond and placed in clean water; (4) chemically identify mutagenic and carcinogenic chemicals in the water; (5) determine if the water was mutagenic; (6) determine if tumors would develop in new fish placed into the final oxidation pond; (7) determine if hepatic-enzyme induction occurred in fish exposure to the water (8) determine if viruses were associated with the papillomas by ultrastructural examination, transmission by cell-free homogenate, and isolation of virus with cell-culture techniques; (9) determine if lesions would develop in brown bullheads exposed during embryonic development to a concentrate of the final oxidation pond water; and (10) determine if tumors would develop on black bullheads exposed to an organic concentrate of the final oxidation pond sediment injected or applied topically to the mouth.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

The Tuskegee, Alabama, sewage treatment plant final oxidation pond had wild black bullheads with a 73% prevalence of oral papillomas. Results of this study indicated that the acidic organic solvent extract of this oxidation pond water was seasonally mutagenic to Salmonella typhimurium bacterial tester strains TA98 and TA100 if activated with Arochlor-induced rat liver enzymes. No evidence of a viral etiology for the papillomas was found during ultrastructural studies of the lesions or during transmission attempts.

Caged black bullheads in the oxidation pond developed oral papillomas in the same mouth location occupied by papillomas in wild black bullheads. The lesions in caged fish were probably related to the mutagenic chemicals in the water because there was also induction of hepatic enzymes in fish exposed to the final oxidation pond water. Neoplasms did not develop in adult fish or embryos exposed to water or sediment extracts.

The usefulness of exposing caged fish to water for detection of chemical carcinogens was demonstrated by the development of neoplasms on caged black bullheads. The use of chemical analysis in this case failed to indicate that mutagenic or carcinogenic chemicals were present. Laboratory exposures of black bullheads and brown bullhead embryos to organic extracts of the Tuskegee wastewater failed to cause tumors. Additional refinement of techniques may improve the usefulness of laboratory exposures, but presently field exposures of caged fish are more conclusive.

Chlorination was implicated in the induction of the papillomas because the prevalence decreased after the chlorination rate was reduced. Further studies should determine the identity of the mutagenic and carcinogenic chemicals, the role of chlorine in causing these tumors, and the persistence of the carcinogenic chemicals after release into the receiving stream.

SECTION 3

STUDY SITE

The 0.8-ha final oxidation pond of the Tuskegee, Alabama, sewage treatment plant was designed for post-secondary treatment of sewage effluent. Water retention time in the pond depends on the flow rate and averages 4-5 days. Sewage treatment before the oxidation pond consists of sedimentation, activated sludge secondary treatment, and disinfection by cholorination (Fig. 1). During January-November 1979, monthly average residual chlorine concentration was 1.2-3.1 mg/liter in the effluent leaving the chlorine contact chamber and when entering the final oxidation pond (Fig. 2). Since November 1979 this chlorine concentration has ranged from 0.3 to 1.2 mg/liter. Residual chlorine concentration of water leaving the final oxidation pond is usually 0.1 mg/liter, approximately the same as before November 1979.

The portion of Tuskegee's sewage that entered this plant contained only domestic wastes; however, following heavy rainfall the flow rate into the plant increased greatly, indicating that surface runoff from surrounding agricultural and residential areas entered the sewage system.

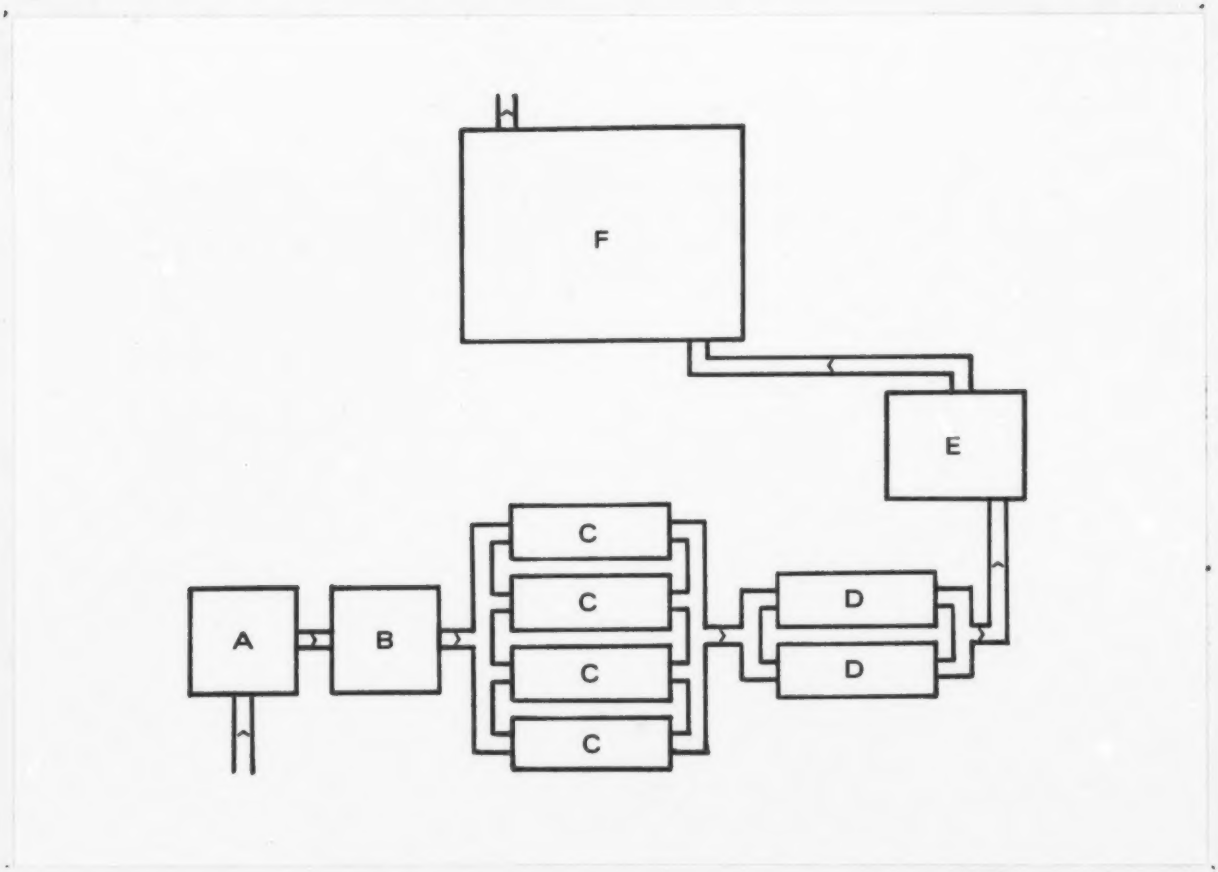


FIGURE 1. Schematic plan of the Tuskegee, Alabama, sewage treatment plant. A, pumping station; B, grit removal; C, aeration basins; D, settling basins; E, chlorine contact chamber; F, final oxidation pond.

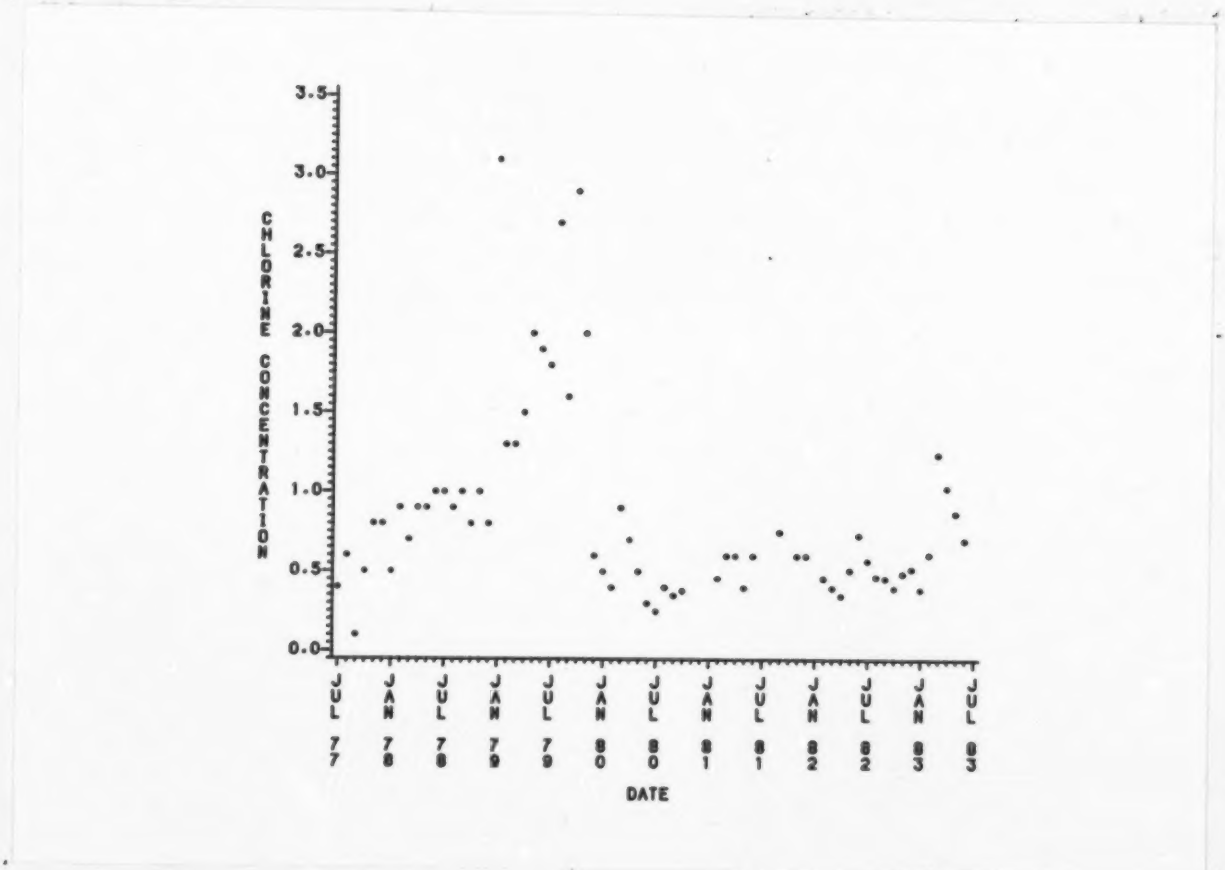


FIGURE 2. Monthly averages for total residual chlorine (mg/liter) in water leaving the chlorine cotact of the Tuskegee sewage treatment plant.

SECTION 4

MATERIALS AND METHODS

Fish were collected from the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant with gill nets and traps. During 2 bacterial epizootics, moribund fish were picked up with a dip net. For determining prevalence of papillomas, most papillomas were diagnosed grossly. Representative lesions were examined by light microscopy to confirm the diagnosis. Fish were put back into the pond if they were not necropsied or used in experiments. The papillomas on 26 adult black bullheads from the final oxidation pond were measured, the fish were identified with tags, and then placed in 3-m diameter plastic pools at the Alabama Agricultural Experiment Station, Auburn, Alabama, to determine the fate of papillomas after the fish were placed in clean water.

For light microscopy, tissues were fixed in 10% buffered formalin or Bouin's, embedded in paraffin, sectioned at 6-um thickness, and stained with hematoxylin and eosin. Selected paraffin sections were also stained with periodic acid-Schiff, Masson's trichrome stain, and Feulgen reaction.

For transmission electron microscopy (TEM), 1-mm^3 pieces of tissue fixed in phosphate buffered glutaraldehyde and post-fixed in osmium tetroxide were embedded in Eopn-Araldite, sectioned at approximately 70-nm thickness, and stained with lead citrate and uranyl acetate.

The Alabama Pesticide Residue Laboratory, Auburn, Alabama, and the Alabama Department of Public Health Laboratory, Montgomery, Alabama, used gas chromatography to determine if toxic organic chemicals were in the water or sediment of the final oxidation pond.

Extracts of the final oxidation pond water were tested for mutagenicity with the Ames test (Ames et al. 1975) using Salmonella typhimurium tester strains TA 98 and TA 100 supplied by Dr. Ames. Positive controls were run with benzo(a)pyrene. Three types of organic extracts of the pond water for mutagenicity testing. For one procedure 10 liters water was filtered through XAD resin and the organics were eluted with acetone. For the other procedures, the pH of 2 liters of water was adjusted to either 1.0 with HCl (for acid fraction) or 12-13 with NaOH (for basic fraction) and then extracted three times with 200 ml of 25% ether and 75% hexane per liter of water. For all procedures, the bulk of the solvents were removed by distillation. The last 20 ml of solvent was evaporated under a stream of nitrogen. Extracts were dissolved in dimethylsulfoxide and filter sterilized before addition to the agar. Concentration of the organic extract ranged from 0.3-3.0 mg/plate.

Black bullheads, brown bullheads (Ictalurus nebulosus), yellow bullheads (Ictalurus natalis), and channel catfish (Ictalurus punctatus) were placed into cages in the final oxidation pond and a control pond at the Alabama Agricultural Experiment Station. The 1-m^3 cages were allowed to rest on the pond bottom (sinking cages) or suspended off the bottom by floats (floating cages). Cages were placed at three locations in the final oxidation pond: near the inlet (inlet A), approximately 60 meters from the inlet (inlet B) or near the pond

outlet (outlet). Most cages were stocked with 50 fish of a single species with total lengths of the fish between 5-10 cm.

Glucuronosyltransferase (UDP-GT) and sulfotransferase (ST) were assayed in livers of caged fish as part of a separate EPA cooperative agreement to Dr. D. R. Strength (Department of Animal and Dairy Sciences, Auburn University). Livers were homogenized in a Potter-Elvehjem-type homogenizer with teflon pestle with 4 parts of 0.25 sucrose containing 0.01M Tris, pH 7.5. A clarified homogenate was prepared by centrifuging at 10,000xg for 30 min. Microsomes were sedimented at 100,000xg for 1 hr, yielding the supernate and microsomal fractions. The microsomes were washed twice in Tris-sucrose buffer, recovered by centrifugation and suspended in 0.1M Tris buffer, pH 7.5 prior to use or storage. Supernate fractions for immediate assay of ST were prepared by homogenizing livers directly in 0.1M Tris buffer, pH 7.5, and centrifuging 1 hr at 100,000xg. Protein concentrations were determined by the procedure of Lowry et al. (1951).

UDP-GT was assayed colorimetrically by the decrease in absorbance at 400 nm using p-nitrophenol as an acceptor. The reaction ixture contained the following in umoles; p-nitrophenol, 0.12; UDP-glucuronic acid, 1.2; $MgCl_2$, 4; Tris buffer, pH 7.5, 11; the total reaction volume of 0.2 ml also contained 40 ug of Triton X-100 and 20 to 50 ug of microsomal protein. The reaction was started by adding UDPGA and stopped after 10 min incubation at 37 C by adding 0.2 ml of ethanol. The reaction mixture was then centrifuged for 10 min at 1,000xg and 0.1 ml of the supernate was added to 0.9 ml of 0.1M EDTA, pH 9.6 and the A_{400} determined. The change in absorbance was determined from the difference in A_{400} of identical reaction mixtures incubated zero and 10 min, respectively; the quantity of p-nitrophenol was ascertained from standard solutions measured in the same medium.

ST activity was determined colorimetrically measuring the p-nitrophenol released from PNPS in the presence of PAP. The reaction mixture from the 100,000xg supernate fraction of tissues in a total volume of 1.0 ml. The reaction was conducted at 37 C for 10 min and was terminated by adding 2.5 ml of ethanol. After centrifugation at 1000xg for 10 min, the supernate was decanted into a tube containing 0.5 ml of 0.2M glycine buffer, pH 10.4 and 1 ml of distilled water (total volume used 5 ml). The absorbance at 400 nm was measured in a spectrophotometer, or alternatively in a Klett-Summerson Colorimeter with #42 filter. Zero time controls and standard solutions of p-nitrophenol were measured in similar fixtures; the p-nitrohenol libertated from PNPS was expressed in nmoles/min/mg protein.

Papillomas from final oxidation pond black bullheads were homogenized in Hank's balance salt solution (HBSS), filtered through a 0.45-um filter, and injected subcutaneously into the mouth fornices of 19 adult black bullheads. Seven control black bullheads were injected with HBSS. Each fish was kept in a separate 40-liter aquarium and examined monthly.

Brown bullhead broodstock were obtained from Easterling's Fish Hatchery, Clio, Alabama. On May 18, 1981, paired broodfish were placed in spawning pens in an earthen pond at the Alabama Agricultural Experiment Station. Spawning boxes were checked for eggs twice daily and eggs were transferred to recirulating aquaria.

Eggs were kept in a recirculating systems with 10, 15-liter aquaria. Filtered pond water was used to fill the system initially and to replace water lost by leakage. Water drained from the aerated aquaria into a lower, 273-liter reservoir and then flowed by gravity to the aquaria. Eggs were incubated in a recirculating system instead of the customary, flow-through troughs to avoid the use of pond water with variable water quality.

Aqueous solutions of afltoxin B_1 (AFB) were prepared from a 500 mg/liter stock solution. To facilitate solubilization, 10 mg AFB (Sigma Co., St. Louis, Mo.) were dissolved in 10 ml dimethylsulfoxide (DMSO) and ethanol. Aflatoxin B_1 was not purified prior to use. DMSO was added as a solvent after it was observed that ethanol alone was insufficient for solubilization. Aflatoxin B_1 concentrations of 0.5, 1.0 and 1.5 mg/liter were made from the stock solution with reconstituted soft water immediately before use.

Organic compounds were extracted from 15 liters of water collected at the outlet from the final oxidation pond of the South Tuskegee Water Pollution Control Plant, Macon County, Alabama, in mid-May 1981. The extraction procedure, a modification of the technique used by Rappaport et al. (1979), used a column of purified XAD-2 end XAD-7 resin. Unfiltered water flowed into the column by gravity at 19 ml/min. The column was dried under nitrogen to remove residual water, and organics were eluted with acetone. The acetone was then dried under a nitrogen stream and the flask containing dried organics was stored at -20 C.

Prior to embryo exposure, the yellowish-brown film of organics was redissolved in 0.2-ml 95% ethanol and 0.4-ml DMSO, and diluted to 200 ml with reconstituted soft water. The final, 200-ml solution was a 75-fold concentrate of the effluent-water organics. Organic yield was not directly measured but estimated to be between 0.7 mg/liter and 1.0 mg/liter (10 and 15 mg for 15 liters) based on later extractions of the same water.

When eggs were five days of age, the egg mass was subdivided into groups of approximately 200 eggs, and each group was placed in 300-ml glass dishes containing 200 ml of the sublethal test solutions for one hour.

After hatching, fish were kept in aquaria until the yolk sac was absorbed. Fish from the first replicate were transferred to 5700-liter plastic pools and fish from the second replicate were kept by treatment group in cages in an earthen pond on the Alabama Agricultural Experiment Station. Fry were fed on a 48% crude protein diet (Silver Cup No. 1), and after four months of age on a 36% crude protein feed (Auburn Feed No. 3).

The only treatments sampled from the first replicate were the 0.5 mg/liter AFB and solvent control, due to accidental drainage of the other plastic pools after stocking. Samples of three to five fish from the two treatments surviving in plastic pools were fixed in Bouin's fluid daily for two weeks after fertilization, and at 25 days, three months, and six months after hatching. Samples of solvent control fish were fixed in Bouin's at these time periods for histological examination of normal liver development and morphology. Eight months after hatching, equal numbers of both the 0.5 mg/liter AFB and the solvent control treatments were sampled as follows: 147 fixed in Bouin's, three

dissected for fixation of livers for electron microscopy, and 20 were held for microsomal preparation of liver tissue.

All but two treatments of the second replicate, placed in cages, were lost to bacterial infections during the induction period. Surviving treatments were the 1.0 mg/liter AFB group and the Tuskegee effluent concentrate group. Brown bullheads of the 1.0 mg/liter AFB treatment were fixed in Bouin's at 25 days (four) and at eight months (twelve) after hatching. Four brown bullheads exposed to the Tuskegee effluent concentrate were fixed in Bouin's 25 days after hatching. Remaining fish of the effluent concentrate treatment were later lost to bacterial infections.

Sediment from the inlet area was collected and allowed to dry for 96 hours. The sediment was then further desiccated with Na_2SO_4. Organics from the sediment were then extracted using acetone and methylene chloride. The extract obtained after distillation was dissolved in DMSO. Six fish were injected in the right mouth fornix with 0.1 ml of the extract at a concentration of 83.3 mg/ml DMSO. Six control fish received an injection of 0.1 ml DMSO. Fish were injected once every 2 months for a period of 9 months. The same extract was also dissolved with DMSO to give a concentration of 50 mg/ml DMSO. The mixture was applied to the entire oral area of 8 black bullheads using a cotton swab. Each fish was swabbed with a circular motion for 1 minute, at weekly intervals, for a period of 6 months. Eight other fish that served as controls received weekly, 1-minute swabs of DMSO only.

SECTION 5

RESULTS

PAPILLOMAS ON WILD BLACK BULLHEADS IN THE FINAL OXIDATION POND

The prevalence of papillomas in wild, adult black bullheads in the final oxidation pond decreased from 73% in 1980 to 23% in March 1983 (Fig. 3). Another change in the papillomas was the size; the large papillomas seen protruding from the mouth of black bullheads in 1979-1980 (Grizzle et al. 1981) were not found in recent samples. All neoplasms were located in the oral cavity or on the lips except for 1 papilloma and 1 dermal fibroma on the dorsal surface of the heads of 2 specimens.

The only other species of fish present in the final oxidation pond before May 1980 were green sunfish (Lepomis cyanellus) and golden shiners (Notemigonus crysoleucas). During May and July 1980, 2,000 silver carp (Hypophthalmichthys molitrix) were released in the final oxidation pond. No tumors were found on any of these species.

The papillomas consisted of hyperplastic nucosa covering papillae of submucosa (Fig. 4). Goblet cells and alarm substance cells were absent from parts of the lesion; in other areas these cells were numerous and formed cell nests (Fig. 5). Cytoplasmic inclusion bodies in cells near the tumor surface were eosinophilic, PAS positive, and weakly Feulgen positive. The number of mitotic figures was similar to that in normal oral mucosa.

Ultrastructurally, the oral papillomas of wild black bullheads from the final oxidation pond had epithelial cells resembling normal oral mucosa cells and a central connective tissue column that was continuous with the submucosa. Near the tumor surface there were mucous cells, and plasma membranes of the epithelial cells adhered closely with those of adjacent cells. Desmosomes occurred less frequently between the cells than in normal mucosa. Mitochondria, endoplasimic reticulum, and ribosomes wee concentrated in the peripheral cytoplasm of the epithlial cells. These surface cells often contained glycogen particles among the tonofilaments. The mid to near basal portion of the papilloma consisted of stellate epithelial cells having more tonofilaments, fewer organelles in the cytoplasm, and more intercellular space than epithelial cells near the surface. Desmosomes occurred more frequently between the cells in the basal area than near the surface. Alarm substances occurred in the mid portion of the epithelium and were surrounded by stellate cells. Numerous lymphocytes were present in the basal region of the tumor. The basal epithelial cells contained numerous mitochondria, endoplasmic reticulum, and ribosomes in the cytoplasm. Plasma membranes adhered to the adjacent cells by desmosomes. Intercellular spaces occurred less frequently in the basal region than in the middle portion of the tumor.

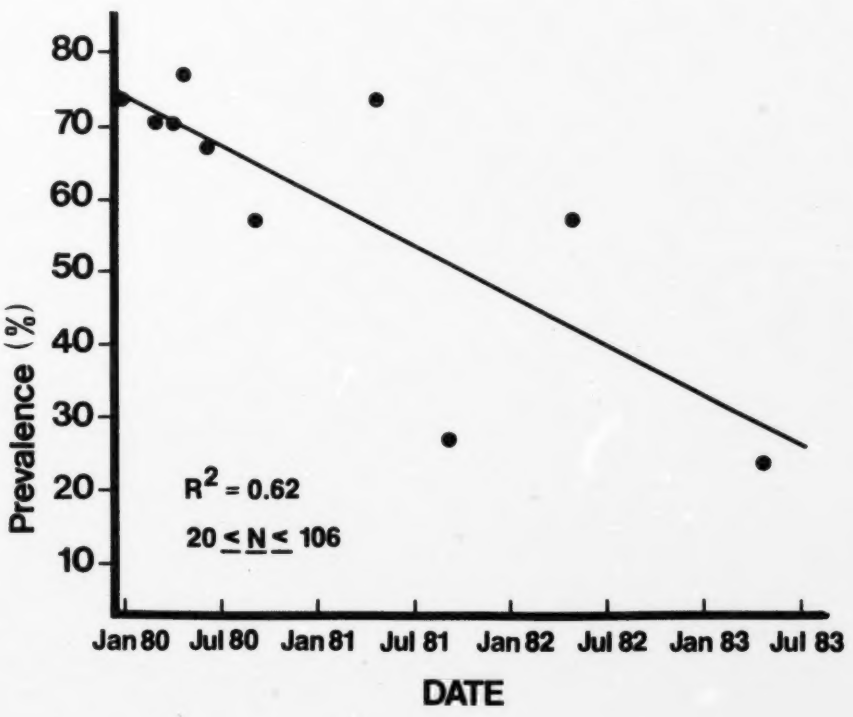


FIGURE 3. Prevalence of papillomas on wild black bullheads from the final oxidation pond of the Tuskegee sewage treatment plant.

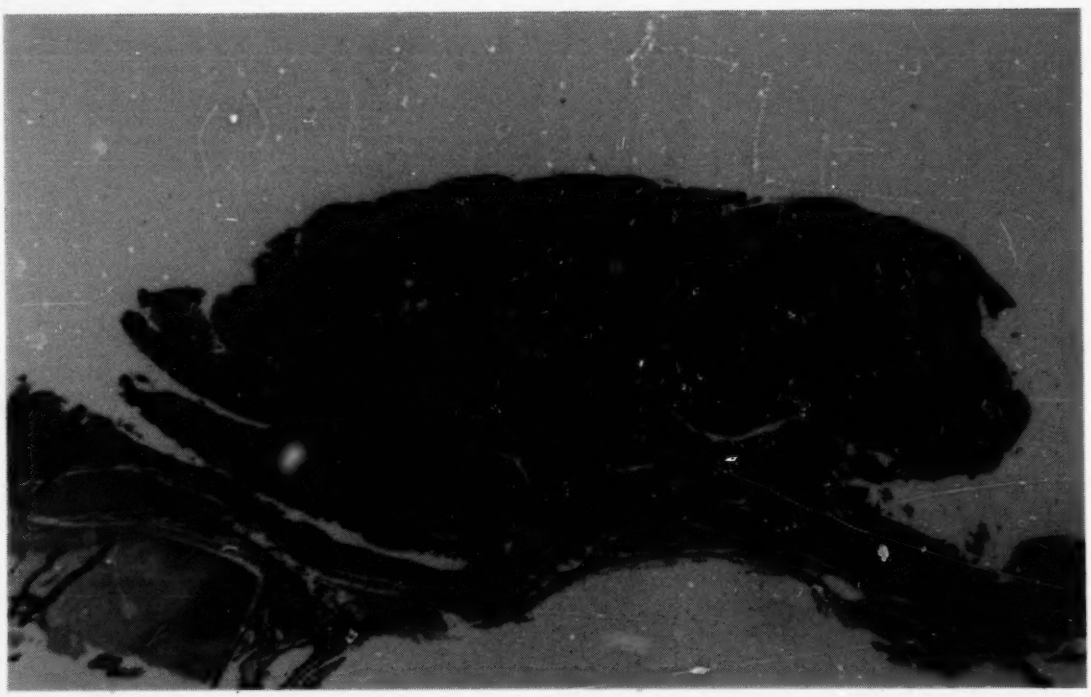


FIGURE 4. Oral papilloma on the mouth fornix of a black bullhead. X20.

Reproduced from
best available copy.

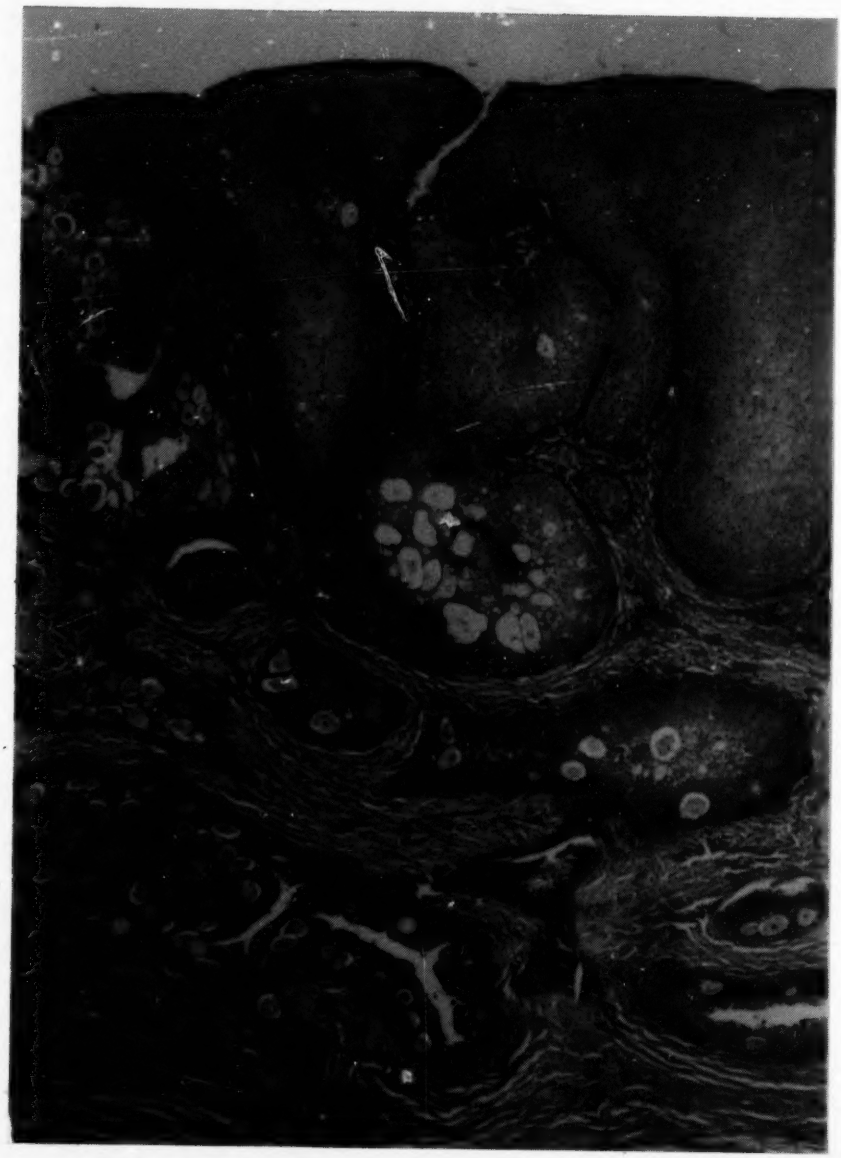


FIGURE 5. Superficial portion of an oral papilloma. Epithelial cells, goblet cells, and alarm substance cells are arranged around lumens forming cell nests. X60.

ANALYTICAL CHEMISTRY OF FINAL OXIDATION POND WATER

Water collected on 16 November, 9 March 1980, 22 January 1982, and 3 March 1982 had unidentified nitrogen and phosphorus organic residues in the 100 ng/liter range. No significant residues of pesticides, herbicides, or PCB were detected in the water or sediment. Water collected on 1 May 1981 contained chloroform (9.0-13.5 ug/liter) and bromodichloromethane (0.7 ug/liter), but chlorodiibromomethane and bromoform were not found with a detection limit of 1.0 ug/liter. Chloroform concentrations were similar before chlorination and in the final oxidation pond.

MUTAGENICITY OF THE FINAL OXIDATION POND WATER

The acid fraction and the XAD extract were mutagenic to Salmonella typhimurium tester strains TA98 and TAI00 only if S-9 (aroclor-induced rat liver enzymes) was present. The mutagenicity of the organic extracts varied seasonally (Fig. 6). The basic fraction was not mutagenic. No differences have been found between extracts prepared by organic extraction of acidified water and extracts prepared by filtering the water through a resin column. Water samples collected at both the inlet and outlet of the final oxidation pond were mutagenic, indicating that the mutagenicity is not lost during retention in the pond.

EXPOSURE OF FISH TO WATER IN THE FINAL OXIDATION POND

Papillomas developed on 5 of the black bullheads kept in cages in the final oxidation pond (Table 1). The papillomas were located in the same area of the mouth and had the same gross and histological appearance as the papillomas on the wild black bullheads in the pond. Four of the papillomas developed between 467-537 days of exposure on fish in a sinking cage near the outlet. One papilloma developed in less than 57 days on a fish in a sinking cage at the inlet B location. The small number of fish developing papillomas prevents conclusions concerning the importance of cage location and contact with the sediment.

The most common types of lesions developing on fish confined to cages in the final oxidation pond were oral mucosa hyperplasia and stomatitis usually located in the mouth fornices. The incidence of oral lesions on caged fish in the final oxidation pond varied depending on the fish species and the year that the fish were exposed (Table 1). The highest incidence was in black bullheads stocked during October 1980 and the incidence was less in each successive stocking. Black bullheads had the highest prevalence of oral lesions of the 4 species tested. Similar oral lesions occurred on control black bullheads (Table 1) but at a prevalence of 9% or less. There were no difference in prevalence of hyperplastic lesions between floating and sinking cages or between inlet and outlet locations.

Table 1. Maximum prevalence of oral lesions on caged fish in the final oxidation pond of the Tuskegee sewage treatment plant. Control is the maximum prevalence of hyperplastic oral lesions in caged fish kept in a control pond. Neoplasms did not occur in control fish.

Species	Stocking Date	Days of Exposure	Hyperplasia		Papillomas			Control
			%	N	%	N	days	%
black bullhead	21 Oct 80	168	95	22	80	5	537	4
	23 Feb 81	43	50	12	0			
	29 Oct 81	407	33	6	0			0
	9 Apr 82	245	25	8	0			
	8 Jul 82	57	17	12	8	12	57	9
yellow bullhead	12 May 81	683	0		0			14
brown bullhead	29 Oct 81	228	0		0			0
channel catfish	16 Jan 81	436	30	10	0			8
	29 Oct 81	513	4	28	0			4
	8 Jul 82	254	0		0			5

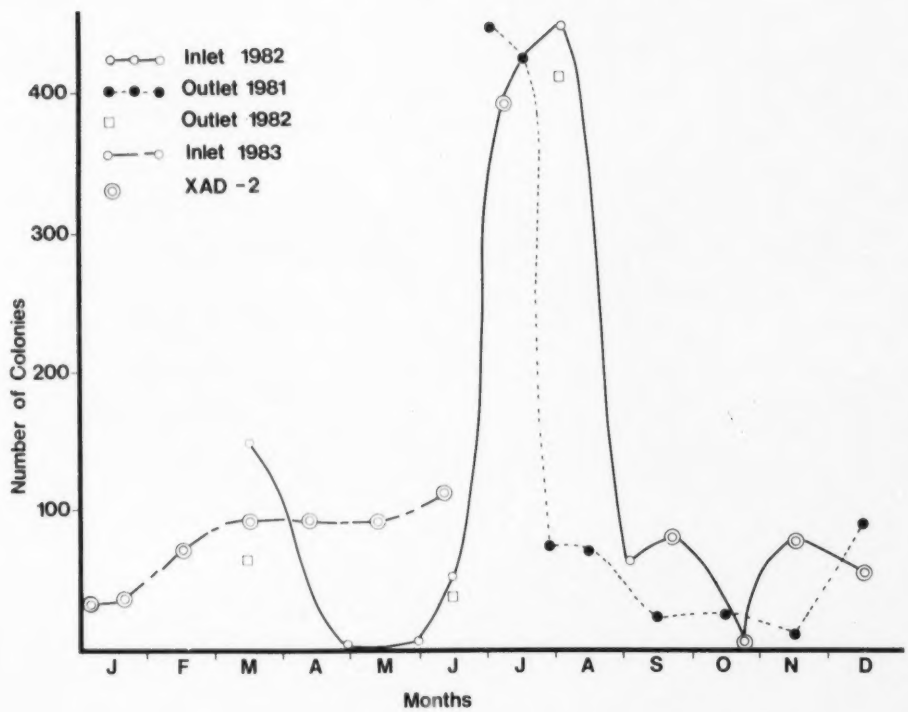


FIGURE 6. Response of Salmonella typhimurium strain TA100 in the Ames test to organic extracts of water collected at the inlet or outlet of the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant. The number of colonies is the difference between test and control plates. The extracts tested were prepared by solvent extraction of acidified water samples or by XAD-2 resin absorption.

INDUCTION OF HEPATIC ENZYME SYSTEMS

UDP-GT in channel catfish in the final oxidation pond was higher than in control channel catfish (Fig. 7). UDP-GT activity in other ictalurid species and ST activity in all species tested in the final oxidation pond were not consistently different than controls.

VIROLOGY OF THE PAPILLOMAS

Examination of papillomas with TEM failed to reveal virus-like structures. Fish injected with homogenized papillomas were observed 11-14 months without signs of papillomas or hyerplastic lesions.

EXPOSURE OF BROWN BULLHEAD EMBROYS TO FINAL OXIDATION POND WATER AND AFB

Trunk kidneys of fish, 25 days after exposure to 1.0 mg/liter AFB, had atrophied glomeruli and renal tublar necrosis. Focal areas of atrophied epithelial cells of renal tubules were dispersed among tubules of normal structure. Livers of fish with renal lesions appeared normal.

Twelve fish from the embryo exposure to 1.0 mg/liter AFB were examined after eight months. Pathological effects were absent in all liver and kidney tissue examined.

Exposure of brown bullhead embryos to 0.5 mg/liter AFB did not cause pathological effects to livers and trunk kidneys at any development stage examined. Liver tissue from a subsample of this group showed normal ultrastructure, with individual variation in glycogen content.

No pathological effects were observed in livers or trunk kidneys of brown bullheads 25 days after embryo exposure to an organic concentrate of Tuskegee effluent water.

LABORATORY EXPOSURES OF BLACK BULLHEADS TO SEDIMENT EXTRACTS

Black bullheads injected with 0.1 ml DMSO developed necrosis at the injection site. Bacterial infections occurred in the black bullheads being swabbed with sediment extract. No tumors developed in either the injected or swabbed fish after 8 months.

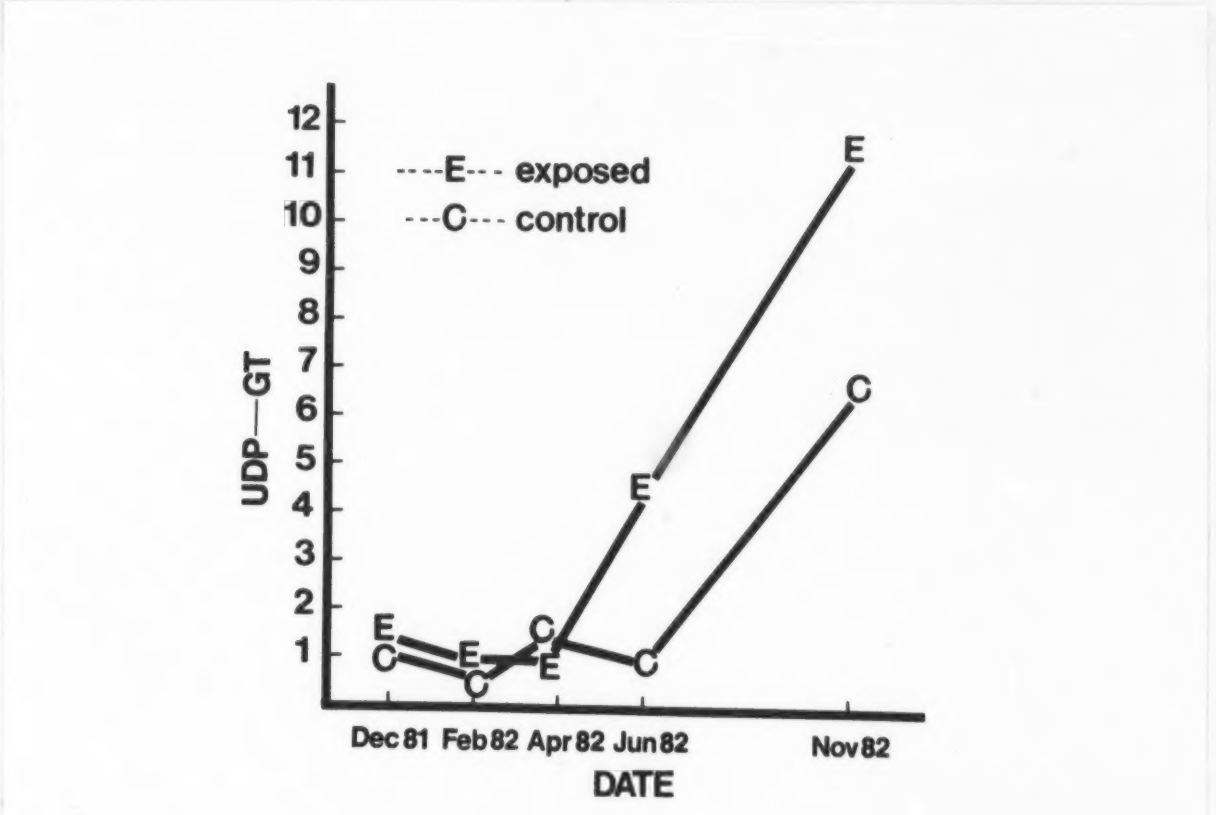


FIG 7. UDP-glucuronosyltransferase (UDP-GT) activity (nmol/min/mg protein) in liver microsomes of caged channel catfish in the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant or in a control pond.

SECTION 6

DISCUSSION

Histologically, the black bullhead papillomas were similar to those of brown bullheads Ictalurus nebulosus described by Lucke and Schlumberger (1941). Harshbarger (1977) considered bullhead papillomas to be a common type of fish neoplasm. However the papillomas in the present case are distinctive because most were uniformly located in the mouth fornices rather than in variable locations.

The neoplastic nature of the papillomas we described is indicated not only by the histopathology but also by their persistence after removal of the fish from the final oxidation pond. If these lesions were inflammatory responses they would have regressed more quickly.

The reason for the uniform location of the papillomas in this population of black bullheads may be related to the presence of the oral valve in the anterior buccal cavity. The oral valve is a thin fold of mucosa located immediately posterior to the upper and lower lips. The folds forming the oral valve terminate laterally near the mouth fornix, and the lateral ends of these folds were often inflammed in fish, including controls, kept in cages during this study. The high incidence of mucosal hyperplasia in the oral valve area also indicates that this location is sensitive to toxicants.

No evidence was found that a virus was associated with the papillomas of black bullheads. This is in contrast to other fish papillomas in which viruses can be seen in electron micrographs (Edwards et al. 1977) or isolated in cell culture (Schwanz-Pfitzner 1976). If a virus is associated with the black bullhead papillomas, they are unlike those previously reported to be present in fish papillomas.

In contrast to the direct-acting mutagens present in the neutral or basic fraction of wastewater from other sewage treatment plants (Saxena and Schwartz 1979, Rappaport et al. 1979, Cumming et al. 1980), the acidic fraction of the Tuskegee sewage effluent was most mutagenic but only after metabolic activation by S-9. This indicates that the primary mutagen in the Tuskegee wastewater is different than in previously reported cases of mutagenic wastewater. Both the activated sludge process (Rappaport et al. 1979) and disinfection with chlorine (Cumming et al. 1980) have been implicated in increasing mutagenicity; both of these factors could be involved in the present case.

The cause of seasonal changes in mutagenicity of the Tuskegee wastewater is unknown but may be related to higher temperatures during chlorination because the peak mutagenicity is during summer. Seasonal variation in wastewater contaminants such as pesticides could also be involved. Accumulation or organic material in filter beds of treatment plants was suggested as a cause of seasonal variation in mutagenicity of drinking water (Grimm-Kibalo 1981). Variation in domestic wastewater mutagenicity could be related to variation in drinking water mutagenicity.

The induction of UDP-GT in channel catfish confined to the final oxidation pond indicates exposure to a toxicnt. Similar induction occurs in rats and fish given oral or intraperitoneal doses of carcinogens. The seasonal changes in UDP-GT activity also strongly indicates the effect of temperature on enzyme induction in fish. The conjugase-enzyme induction in channel catfish but not in black bullheads may be related to the greater susceptibility of black bullheads to the tumor inducing effects of chemical carcinogens in the final oxidaiton pond.

The usefulness of exposing caged fish to water for detection of chemical carcinogens was demonstrated by the development of neoplasms on caged black bullheads. The use of chemical analysis in this case failed to indicate that mutagenic or carcinogenic chemicals were present. Laboratory exposures of black bullheads and brown bulhead embryos to organic extracts of the Tuskegee wastewater failed to cause tumors. Additional refinement of techniques may improve the usefulness of laboratory exposures, but presently field exposures of caged fish are more conclusive.

The role of chlorine in causing the black bullhead papillomas was indicated by the reduced prevalence of tumors after the chlorination rate was reduced. Additional study of the Tuskegee, Alabama, sewage treatment plant is warranted to determine the identity of the carcinogenic chemicals and the relationship between chlorination and tumors.

REFERENCES

Ames, B. N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mut. Res. 31:347-364.

Cooper, R. C., and C. A. Keller. 1969. Epizootiology of papillomas in English sole, Parophrys vetulus. Natl. Can. Inst. Monograph 31:173-186.

Cumming, R. B., N. E. Lee, L. R. Lewis, J. E. Thompson, and R. L. Jolley. 1980. Relationship of disinfection to mutagenicity in wastewater effluents. Pages 881-898 in R. L. Jolley, W. A. Brungs, R. B. Cumming, and V. A. Jacobs. Water chlorination environmental impact and health effects volume 3. Ann Arbor Science Publishers Inc., Ann Arbor, Michigan.

Dawe, C. J., and J. C. Harshbarger. 1975. Neoplasms in feral fishes: their significance to cancer research. Pages 871-894 in W. E. Ribelin and G. Migake, editors. The pathology of fishes. University of Wisconsin Press, Madison, Wisconsin.

Edwards, M. R., W. A. Samsonoff, E. J. Kuzia. 1977. Papilloma-like viruses from catfish. Fish Health News 6:94.

Grimm-Gibalo, S. M., B. A. Glatz, and J. S. Fritz. 1981. Seasonal variation of mutagenic activity in drinking water. Bull. Environm. Contam. Toxicol. 26:188-195.

Grizzle, J. M., T. E. Schwedler, and A. L. Scott. 1981. Papillomas of black bullheads, Ictalurus melas (Rafinesque), living in a chlorinated sewage pond. Journal of Fish Diseases 4:345-351.

Grizzle, J. M. and P. Melius. 1983. Causes of papillomas on fish living in chlorniated sewage effluent. United States Environmental Protection Agency EPA-600/S3-82-087, Gulf Breeze, Florida.

Harshbarger, J. C. 1977. Role of the registry of tumors in lower animals in the study of environmental carcinogenesis in aquatic animals. Ann. NY Acad Sci. 298:280-289.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protection measurement with Folin phenol reagent. J. Biol. Chem. 193:265-275.

Lucke, B. and H. G. Schlumberger. 1941. Transplantable epithelioma of the lip and mouth of catfish. I. pathology. Transplantation to anterior chamber of eye and into cornea. J. Exp. Med. 74:397-408.

McAllister, P. E., T. Nagabayaski, and K. Wolf. 1977. Viruses of eels with and without stomatopapillomas. Ann. NY Acad. Sci. 298:233-244.

Rappaport, S. M., M. G. Richard, M. C. Hollstein, and R. E. Talcott. 1979. Mutagenic activity in organic wastewater concentrates. Environ. Sci. and Tech. 13:957-961.

Russell, F. E., and P. Kotin. 1957. Squamous papilloma in the white croaker. J. Natl. Can. Inst. 18:857-861.

Saxena, J. and D. J. Schwartz. 1979. Mutagens in wastewaters renovated by advanced wastewater treatment. Bull. Environm. Contam. Toxicol. 22:319-326.

Schwanz-Pfitzner, I. 1976. Further studies of eel virus (Berlin) isolated from the blood of eels (Anguilla anguilla) with skin papilloma. Prog. Exp. Tumor Res. 20:101.

Siciloiano, M. J., A. Perlmutter, and E. Clark. 1971. Effect of sex on the development of melanoma in hybrid fish of the genus Xiphophorus. Can. Res. 31:725-729.

Sonstegard, R. A. 1977. Environmental carcinogenesis studies in fishes of the Great Lakes of North America. Ann. NY Acad. Sci. 298:261-269.

Tan, B., P. Melius, and J. M. Grizzle. 1981. Hepatic enzymes and tumor histopathology of black bullheads with papillomas. Pages 377-386 in M. Polynuclear Aromatic Hydrocarbons. 5th International Symposium. Battelle Press, Columbus, Ohio.

Vielkind, J., U. Vielkind, and F. Anders. 1971. Melanotic and amelanotic melanomas in xiphorphorin fish. Can. Research 31:868-875.